

## Supporting Information

### **Ultrathin Two-Dimensional Covalent Organic Framework Nanoprobe for Interference-Resistant Two-Photon Fluorescent Bioimaging**

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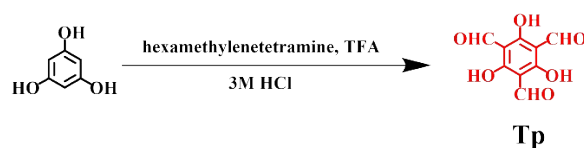
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## Section S-1: Materials and instruments

All chemicals were obtained from commercial suppliers and used without purification. Ultrapure water was utilized from a Milli-Q reference system (Millipore). The field emission scanning electron microscope (SEM) results were obtained on JSM-6700F (JEOL). Transmission electron microscope (TEM) images were recorded using JEM-2100F (JEOL). The atomic force microscopy (AFM) images were gained on a Multimode 8 (Bruker) with ScanAsyst. Powder X-ray diffraction (PXRD) patterns were recorded on Phillips PANalytical diffractometer for Cu K $\alpha$  radiation ( $\alpha = 1.5406 \text{ \AA}$ ), with a scan speed of  $1^\circ \text{ min}^{-1}$  and a step size of  $0.02^\circ$  in  $2\theta$  and Rigaku MicroMax 007HF diffractometer respectively. Samples were observed using a continuous  $2\theta$  scan from  $1.0$ - $40^\circ$ . Fourier transform infrared (FTIR) spectra were recorded on TENSOR27 spectrometer (Bruker) in the  $500$ - $4000 \text{ cm}^{-1}$ . Dynamic light scattering (DLS) measurements were made on a Zetasizer 3000Hs (Malvern). The UV-vis absorption spectra were collected on a UV-2450 UV-vis spectrometer (Shimadzu). Fluoromax-4 spectrofluorometer (HORIBA JobinYvon) was used to collect the fluorescence signal with the slits set at  $5.0 \text{ nm}$ . One-photon (OP) and two-photon (TP) fluorescence bioimages of live tumor cells and tissues were picked up through an Olympus FV1000-MPE multiphoton laser scanning confocal microscope (Olympus).

## Section S-2: Experimental Section

**Synthesis of 1,3,5-triformylphloroglucinol (Tp):** 1,3,5-Triformylphloroglucinol was synthesized according to a previous literature (Scheme S1).<sup>1</sup> Briefly, hexamethylenetetramine (7.4 g, 52.8 mmol) and dried phloroglucinol (3.0 g, 23.8 mmol) were mixed in two-neck round-bottom flask and trifluoroacetic acid (TFA, 45 mL) was added with ice-bath under nitrogen atmosphere. The solution was then allowed to reflux at 100 °C for 2.5 h. After that time period, 3 M HCl (100 mL) was added slowly and the solution was further refluxed for another 1 h. After cooling to room temperature, the solution was filtered through Celite. Obtained filtrate was extracted with dichloromethane ( $4 \times 100$  mL) and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . The obtained extract was concentrated and purified by using hot ethanol to yield an off-white powder.



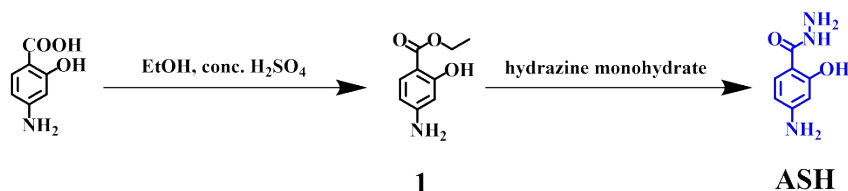
**Scheme S1.** Synthetic scheme of Tp.

**Synthesis of 4-Aminosalicylhydrazide (ASH):** 4-Aminosalicylhydrazide was synthesized according to a previous literature with a two-step reaction (Scheme S2).<sup>1</sup>

**Synthesis of 1:** 4-Aminosalicylic acid (5 g, 32.7 mmol) was dissolved in dried ethanol (50 mL) and conc.  $\text{H}_2\text{SO}_4$  (3 mL) was added dropwise with ice-bath. Then the resultant mixture was refluxed for 3 h under nitrogen atmosphere. After cooling to room temperature, about 30 mL of ethanol was evaporated and the obtained solution was extracted with dichloromethane ( $3 \times 30$  mL) and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . The compound 1 was collected by evaporation of dichloromethane under vacuum.

**Synthesis of ASH:** The compound 1 (2.5 g, 13.8 mmol) was added to about 10 mL of 80% hydrazine monohydrate and then the resultant mixture was refluxed for overnight under nitrogen atmosphere. After evaporation of excess hydrazine monohydrate, the

product was added to ice cold distilled water, followed by extraction in ethyl acetate. The compound ASH was collected by evaporation of ethyl acetate under vacuum.



**Scheme S2.** Synthetic scheme of ASH.

**Synthesis of fluorescent probe NPHS:** The compound NPHS was synthesized according to our optimized synthetic methodology with a three-step reaction (Scheme S3).

**Synthesis of 2:** 4-Bromo-1,8-naphthalicanhydride (2.77 g, 10 mmol) was dissolved in DMF (60 mL) and sodium azide (1.95 g, 30 mmol) was dissolved in water (2 mL). The reaction mixture was protected from light and heated to 80 °C for 2 h, and the solution was poured into ice water. The precipitated yellow solid was filtered to give the compound 2.

**Synthesis of 3:** The reaction mixture of compound 2 (0.239 g, 1 mmol), 4-aminobutanoic acid (0.412 g, 4 mmol), ethanol (30 mL), water (6 mL) was protected from light and heated to 90 °C for 3 h. The mixture was filtered, then dried in the oven to give the compound 3.

**Synthesis of NPHS:** To a DMF solution (10 mL) of compound 3 (0.324 g, 1 mmol) and EDC·HCl (0.250 g, 1.3 mmol), NHS (0.250 g, 1.3 mmol) were added at room temperature. Activation of compound 3 was carried out for 4 h under dark condition, then 3-aminopropyltriethoxysilane (APTES, 0.442 g, 2 mmol) was added and the reaction mixture was stirred for overnight under dark. The crude product was purified by preparative thin layer chromatography to yield pure NPHS.



**Scheme S4.** Synthetic scheme of CNPHS.

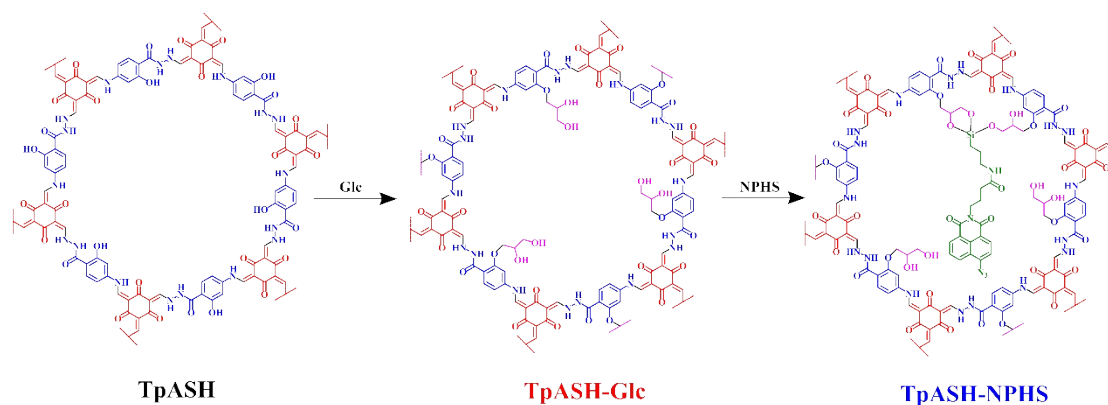
**Synthesis of COFs and Post-synthetic Modifications:** The COF TpASH was synthesized according to a previous literature by salt-mediated Schiff base condensation reaction (Scheme S5).<sup>1</sup> The post-synthetic modifications were carried out on TpASH *via* two step to yield bulk TP fluorescent COF TpASH-NPHS (Scheme S5).

**Synthesis of TpASH:** ASH (75.15 mg, 0.45 mmol) and *p*-toluenesulphonic acid (PTSA) (500 mg) were mixed in a pestle mortar for about 10 min to obtain a sticky salt. And then Tp (63 mg, 0.3 mmol) and de-ionized water were added and mixed. The mixture was then transferred to a glass vial and kept in 90 °C for 12 h. After cooling to room temperature, the resultant product was washed extensively with hot water, DMAc and acetone respectively. The product was dried at 90 °C for overnight to obtain brown

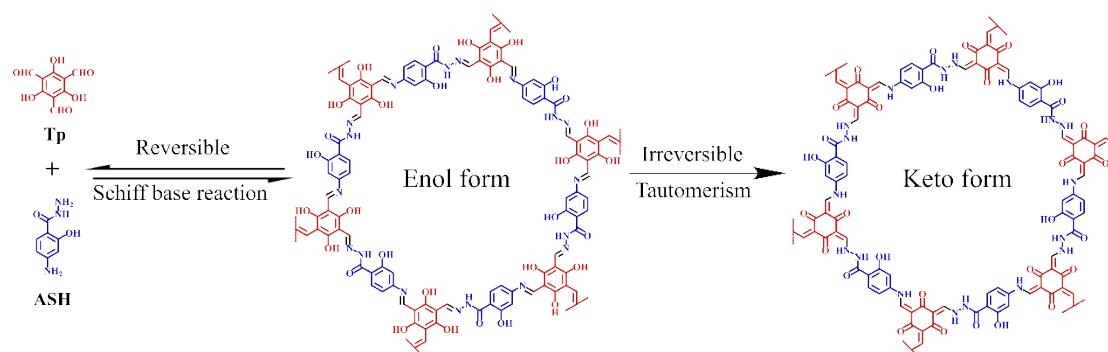
colored COFs.

**Synthesis of TpASH-Glc:** TpASH-Glc was synthesized according to a previous literature.<sup>1</sup> Briefly, TpASH (60 mg) was dispersed in 0.1 M NaHCO<sub>3</sub> solution (25 mL) for 5 min by sonication and glycidol (Glc, 750  $\mu$ L) was added and the reaction mixture was kept under reflux for 24 h. After cooling to room temperature, the resultant product was washed extensively with ethanol and dried at 60 °C for overnight.

**Synthesis of TpASH-NPHS:** TpASH-Glc (50 mg) was dispersed in toluene (25 mL) by sonication under nitrogen atmosphere and NPHS (50 mg) was added. Then the reaction mixture was refluxed under dark for overnight. After cooling to room temperature, the resultant product was washed extensively with ethanol, DMAc and acetone respectively. Finally the product was dried at 60 °C for overnight.



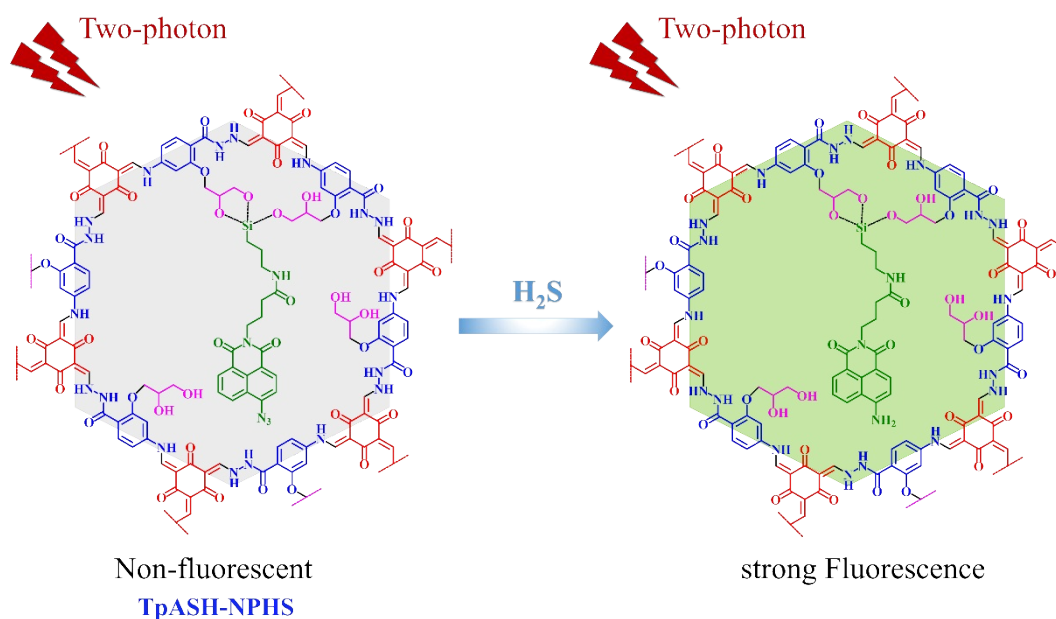
**Scheme S5.** Schematic representation of the post-synthetic fluorophore conjugation.



**Scheme S6.** Schematic representation of the synthesis of TpASH by the combined reversible and irreversible reaction of TP and ASH. The total reaction proceeds in two steps: (1) reversible Schiff-base reaction and (2) irreversible enol-to-keto tautomerism.

**Preparation of Nanoscale TpASH-NPHS:** The nanoscale TpASH-NPHS was prepared by liquid exfoliation of corresponding bulk COFs.<sup>3</sup> In detail, 10 mg of the obtained bulk TpASH-NPHS was dispersed in 20 mL de-ionized water and sonicated with a 6 mm sonic tip (ultrasonic frequency: 20-25 kHz) for 6 h (period of 2 s with the interval of 2 s) using a power of 130 W. The ice-bath was used to keep the system in a relatively low temperature. After natural sedimentation overnight, the suspension of nanoscale TpASH-NPHS was concentrated by centrifugation at 7000 rpm for 5 min.

**Fluorescence Sensing of TpASH-NPHS.** Unless otherwise noted, all spectrophotometric measurement experiments were carried out in 10 mM phosphate buffered solution (PBS, pH = 7.4). The NaHS solution were added into the TpASH-NPHS solution (100  $\mu\text{g/mL}$ ) at a final concentration ranging from 1  $\mu\text{M}$  to 5 mM with incubation for 40 min and then the fluorescence emission spectra was recorded at an excitation wavelength of 440 nm. Both the excitation and emission bandwidths were 5.0 nm. The time-dependent fluorescence spectra of TpASH-NPHS response to  $\text{H}_2\text{S}$  was investigated from 0 to 80 min at intervals of 5 min.



**Scheme S7.** The response mechanism of TpASH-NPHS to  $\text{H}_2\text{S}$ .



**Cell Culture and Cytotoxicity Assay.** The cells (HeLa or HepG2) were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin) at 37 °C in a humidified incubator containing 5% CO<sub>2</sub>. A standard MTS assay was used to assess the cytotoxic of TpASH-NPHS. The cells were seeded at 5×10<sup>3</sup> cells per well in 96-well plates and grown for 24 h under above conditions. Then the cells were incubated with TpASH-NPHS at varied concentrations (10, 25, 50, 100 and 200 µg/mL, respectively) for another 24 h. After washing with Dulbecco's Phosphate Buffered Saline (DPBS) for three times, the standard MTS solution was added to each well and incubated for 0.5 h. Then the cells were used a multimode microplate to determine the cell viability.

**Imaging of H<sub>2</sub>S in Live Cells.** The cells (HeLa or HepG2) were first incubated with DMEM culture medium in a 30 mm optical culture dish for 24 h at 37 °C, then culture medium was replaced and TpASH-NPHS (100 µg/mL) was added into the fresh culture medium for incubating another 2 h at 37 °C. After washing with DPBS for three times, the cells were incubated with different concentrations of NaHS solution for additional 40 min at 37 °C. After washing with DPBS for three times, OP and TP excited fluorescence imaging of the cells was recorded with magnification at 60 ×. The TP images were collected at 460-560 nm upon excitation at 820 nm with femtosecond pulses. The OP images were collected at 500-600 nm upon excitation at 405 nm.

**Endocytosis pathway detection.** The HepG2 cells were incubated with DMEM for 24 h. Then, serum-free DMEM as the control and one of the following inhibitors including chlorpromazine hydrochloride (5 µg/mL), genistein (50 µg/mL), or methyl-β-cyclodextrin (5 mg/mL) dissolved in serum-free DMEM were used to pretreat the cells at 37 °C for 2 h. Then, the medium in each well was discarded and replaced with fresh medium containing TpASH-NPHS (100 µg/mL) and the corresponding inhibitor with incubation at 37 °C for another 2h. And 4 °C culturing was used to inhibit energy-

dependent mechanisms. After washing with DPBS for three times, the cells were incubated with NaHS solution for additional 40 min.

**Imaging of H<sub>2</sub>S in Deep Tissues.** The HeLa and HepG2 tumor tissues slices with a thickness of 500  $\mu$ m were prepared from nude mice. The tissue slices were incubated with TpASH-NPHS at 37 °C for 2 h. Washed with DPBS three times, the tissue slices were co-incubated 100  $\mu$ M NaHS solution at 37 °C for additional 40 min. The tissue slices were incubated without NaHS as control. Then the TP fluorescence microscopy images were collected after washed with DPBS three times.

**Hypoxia Treatment.** Hypoxia treatments were carried out in the AnaeroPack system for cell culture (Mitsubishi Gas Chemical Co., Tokyo, Japan). For experiments at <0.1% O<sub>2</sub> cells were incubated on optical culture dish. Unless otherwise specified, CNPHS (1  $\mu$ M) and TpASH-NPHS (100  $\mu$ g/mL) was utilized under hypoxia for 3h.

**The Selectivity of TpASH-NPHS and CNPHS.** The selectivity of TpASH-NPHS and CNPHS (1  $\mu$ M) was treated with potential intracellular coexisting species, including Cl<sup>-</sup> (1 mM), Br<sup>-</sup> (1 mM), I<sup>-</sup> (1 mM), HCO<sub>3</sub><sup>-</sup> (1 mM), HPO<sub>4</sub><sup>2-</sup> (1 mM), OAc<sup>-</sup> (1 mM), NO<sub>2</sub><sup>-</sup> (1 mM), SO<sub>4</sub><sup>2-</sup> (1 mM), SO<sub>3</sub><sup>2-</sup> (100  $\mu$ M), HSO<sub>3</sub><sup>-</sup> (100  $\mu$ M), S<sub>2</sub>O<sub>3</sub><sup>2-</sup> (100  $\mu$ M), S<sub>2</sub>O<sub>5</sub><sup>2-</sup> (100  $\mu$ M), GSH (1 mM), Cys (1 mM), Hcy (1 mM), ClO<sup>-</sup> (100  $\mu$ M), H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) and NaHS (100  $\mu$ M). The concentration of HepG2 cell lysate were 1 $\times$ 10<sup>4</sup> cells/ $\mu$ L acquired by cell lysis buffer.

**Liver cirrhosis induction in mouse model.** 36 Female Balb/c mice were randomly divided into 6 groups: control group (Normal) and liver cirrhosis group 3-Day, 5-Day, 10-Day, 15-Day and 22-Day. Liver cirrhosis was induced by CCl<sub>4</sub> injection as described previously. Briefly, mice were subcutaneously injected with 40% concentration of CCl<sub>4</sub> diluted with cotton seed oil (the first dose was 0.5 mL/100 g body weight and then 0.3 mL/100 g subsequently every other day). In the meantime, the mice

were maintained with high fat and cholesterol feeds and 15% ethanol in water as drinking water. The control group (Normal) was given the same dose of cotton seed oil with a standard breeding method. All living cells and live animal operations were in accord with institutional animal use and care regulations, according to protocol No. SYXK (Xiang) 2008-0001, approved by Laboratory Animal Center of Hunan.

### Section S-3: Characterization of compounds and COFs

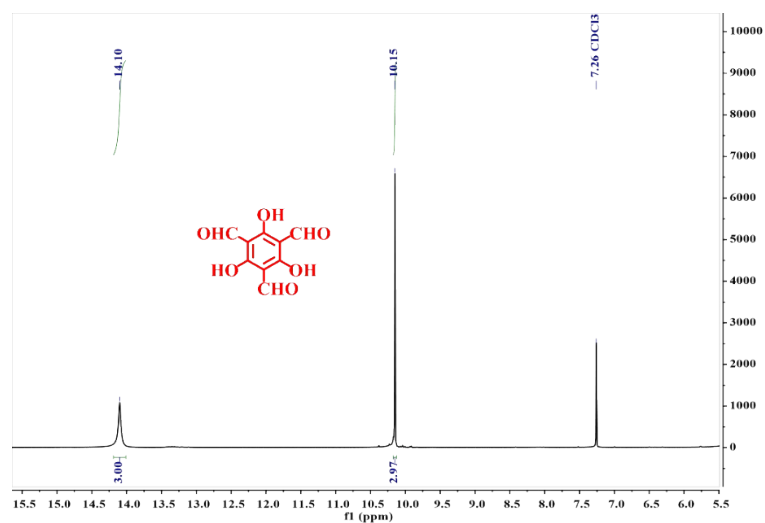


Fig. S1. <sup>1</sup>H NMR spectrum of Tp.

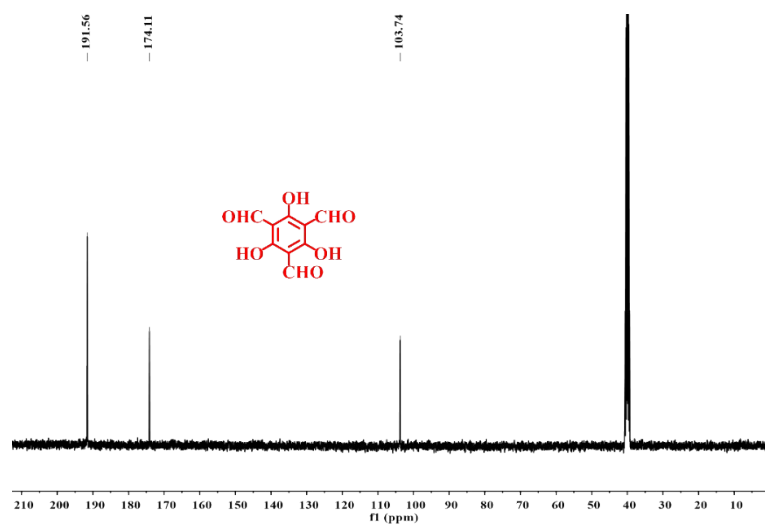
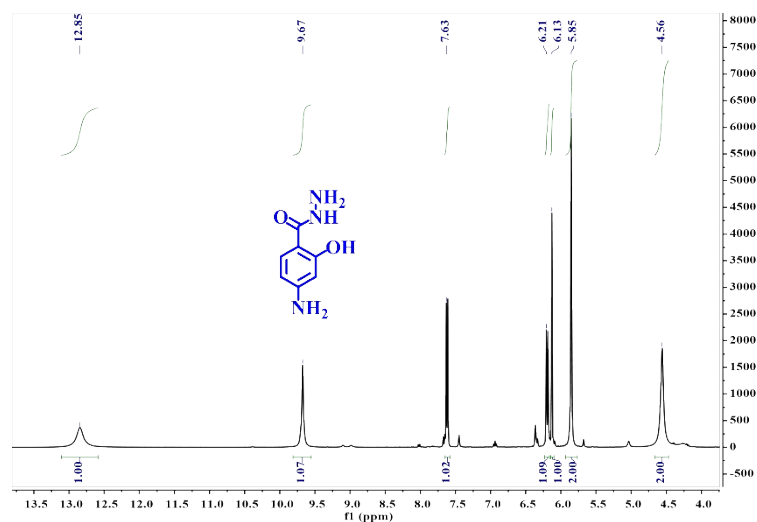
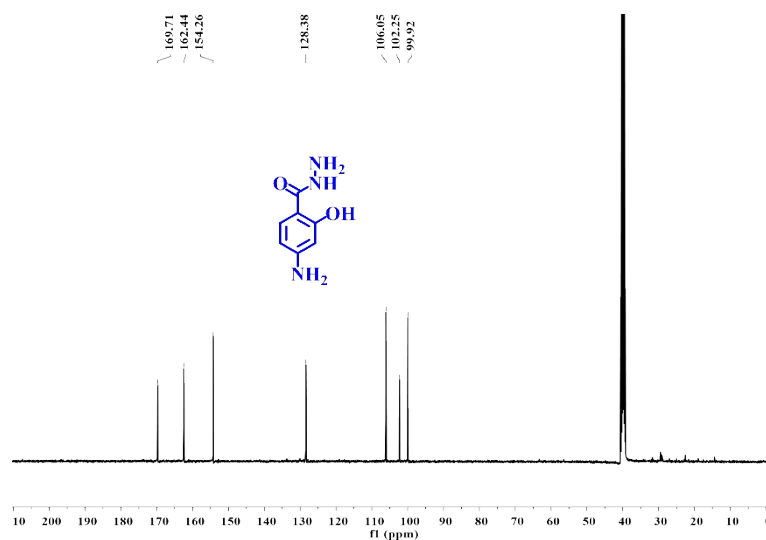


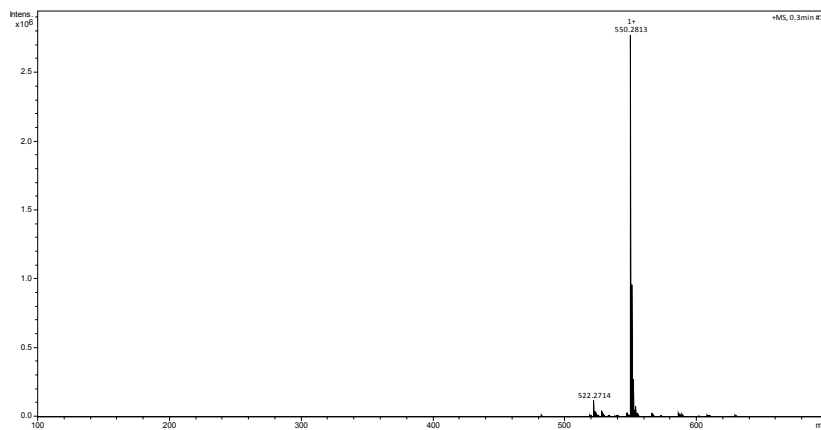
Fig. S2. <sup>13</sup>C NMR spectrum of Tp.



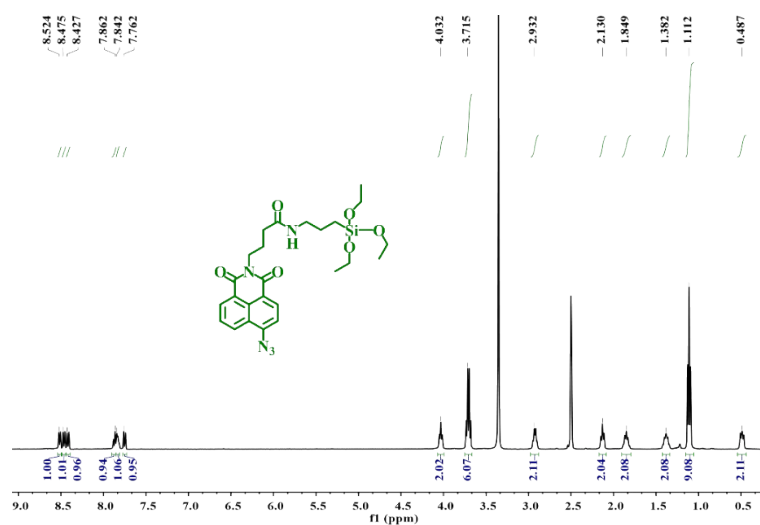
**Fig. S3.**  $^1\text{H}$  NMR spectrum of ASH.



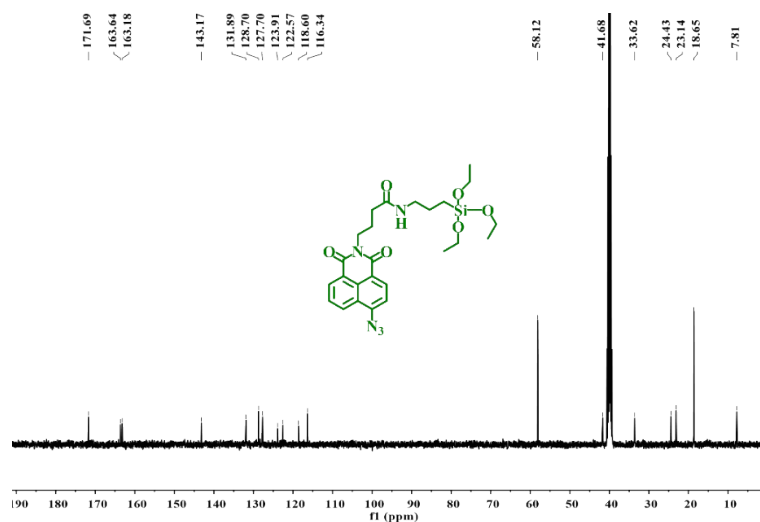
**Fig. S4.**  $^{13}\text{C}$  NMR spectrum of ASH.



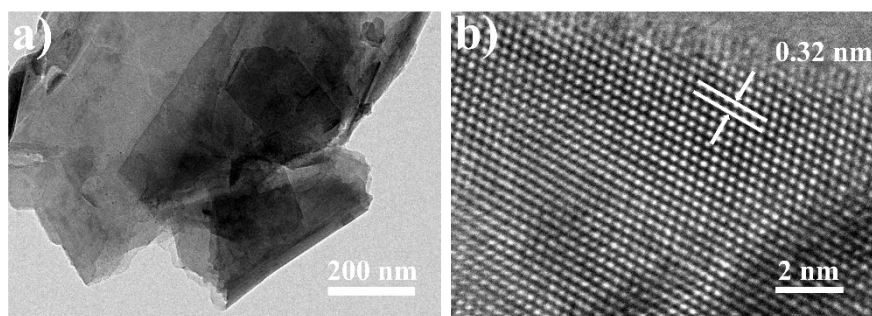
**Fig. S5.** ESI-MS spectrum of NPHS.



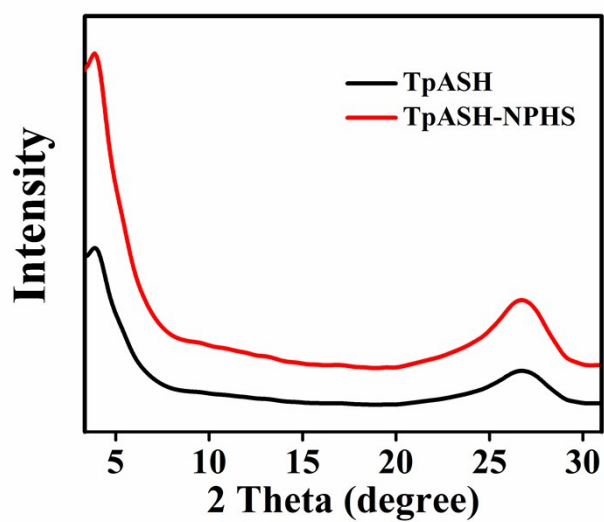
**Fig. S6.** <sup>1</sup>H NMR spectrum of NPHS.



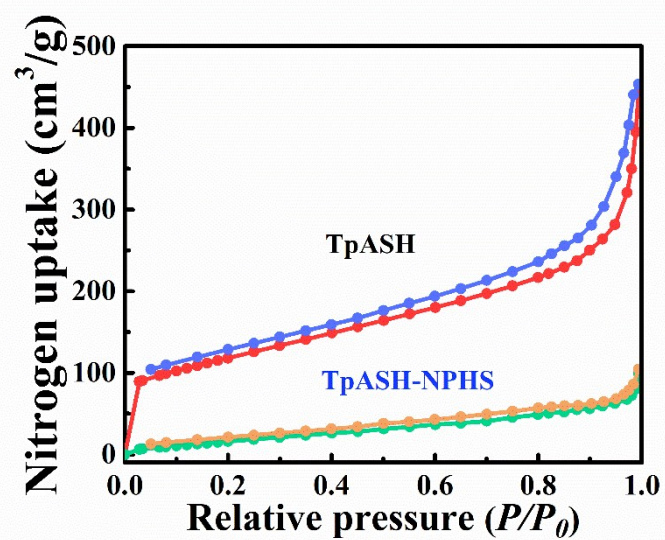
**Fig. S7.** <sup>13</sup>C NMR spectrum of NPHS.



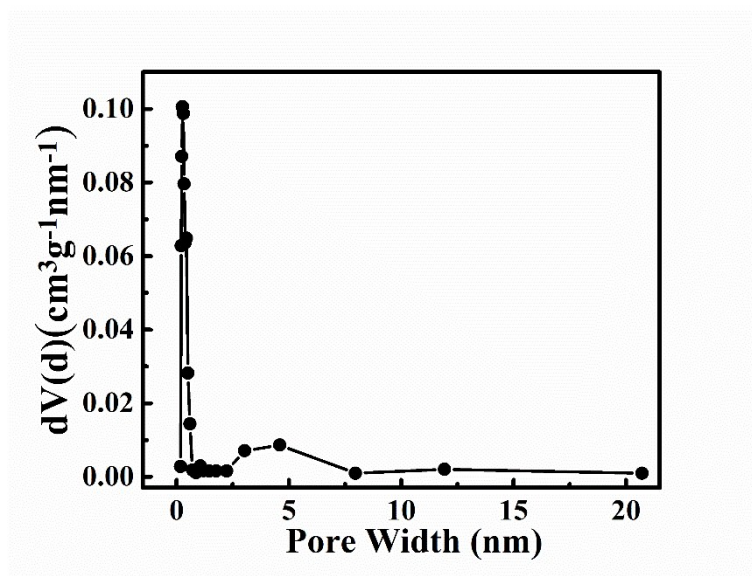
**Fig. S8.** a) TEM and b) HRTEM image of TpASH.



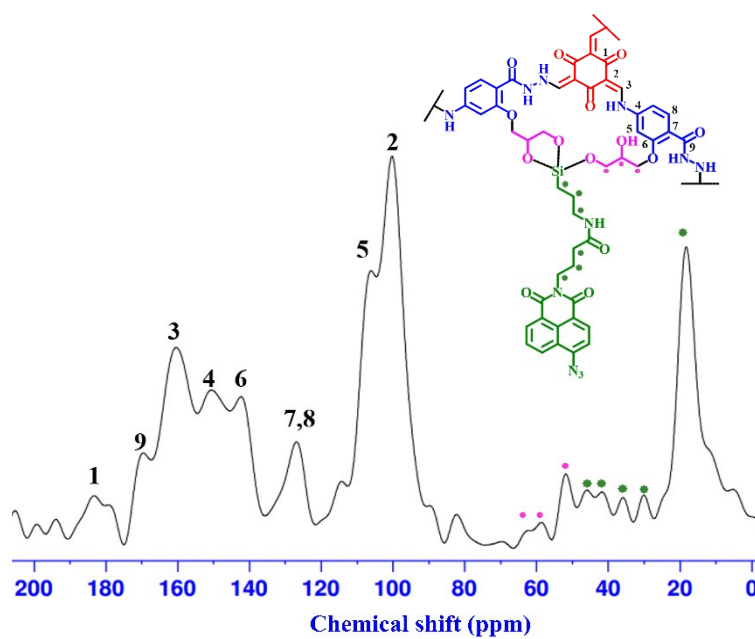
**Fig. S9.** PXRD pattern of TpASH and TpASH-NPHS.



**Fig. S10.** Gas sorption measurements for TpASH and TpASH-NPHS.

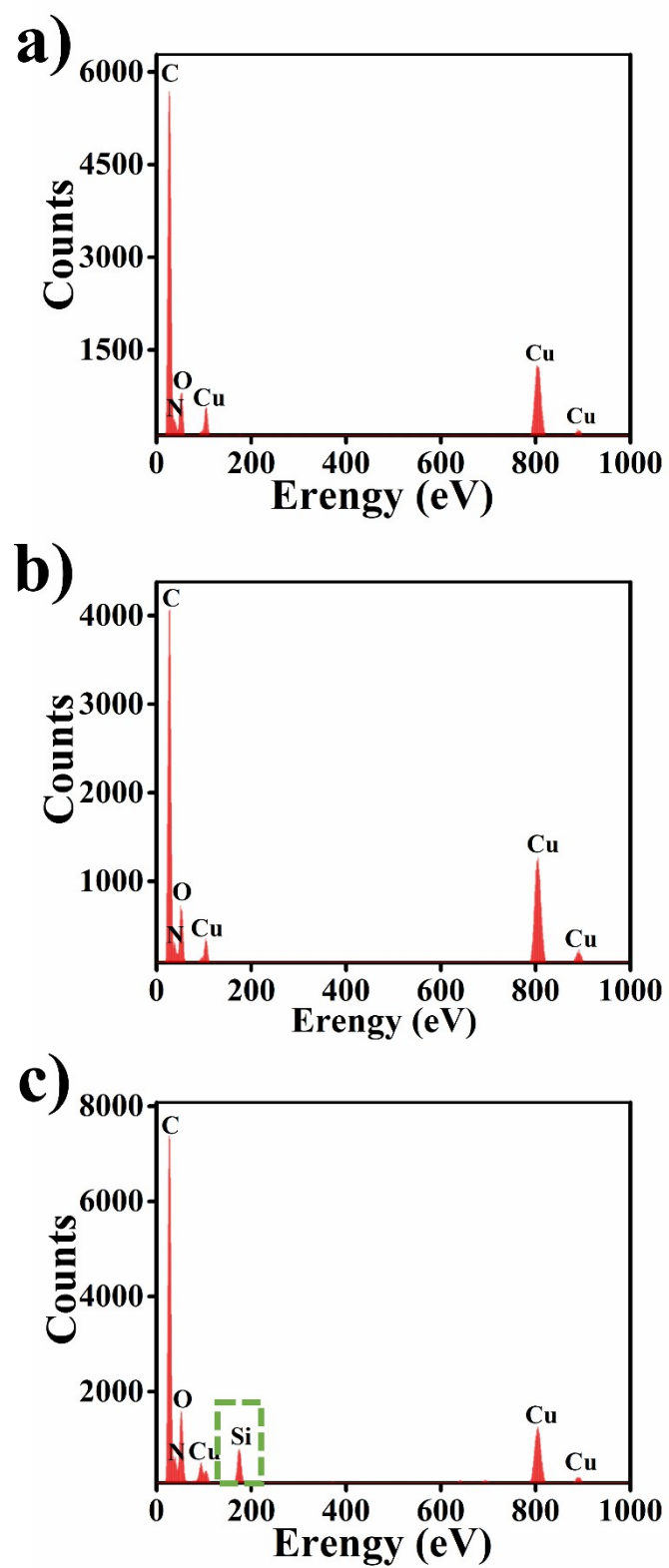


**Fig. S11.** Pore size distribution of TpASH-NPHS.

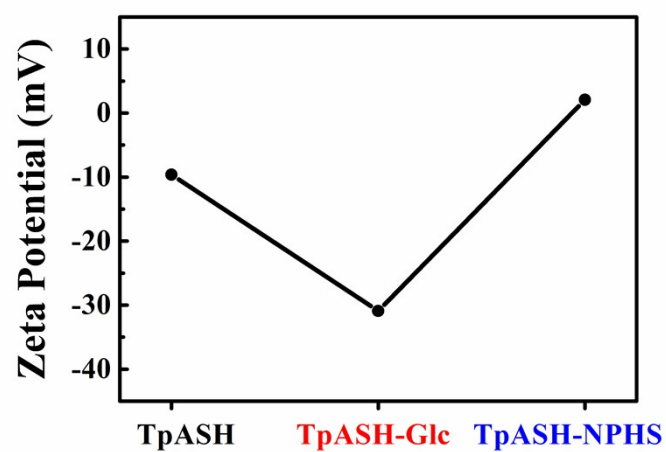


**Fig. S12.**  $^{13}\text{C}$  CP/MAS solid state NMR spectra of TpASH-NPHS.

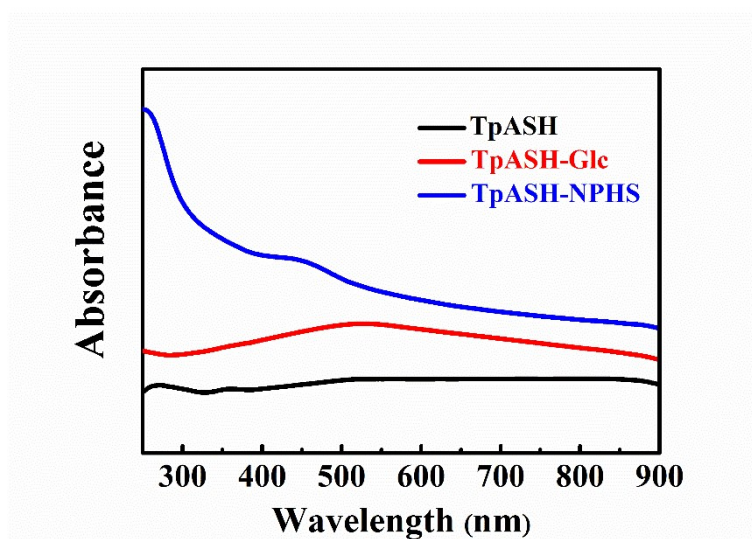




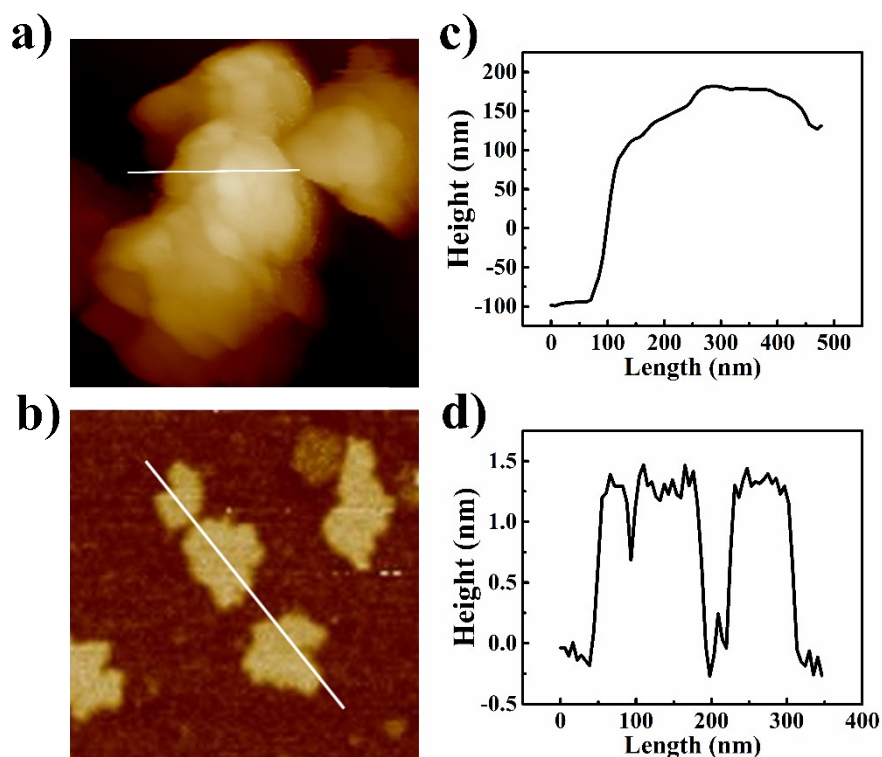
**Fig. S13.** EDX analysis of a) TpASH, b) TpASH-Glc and c) TpASH-NPHS.



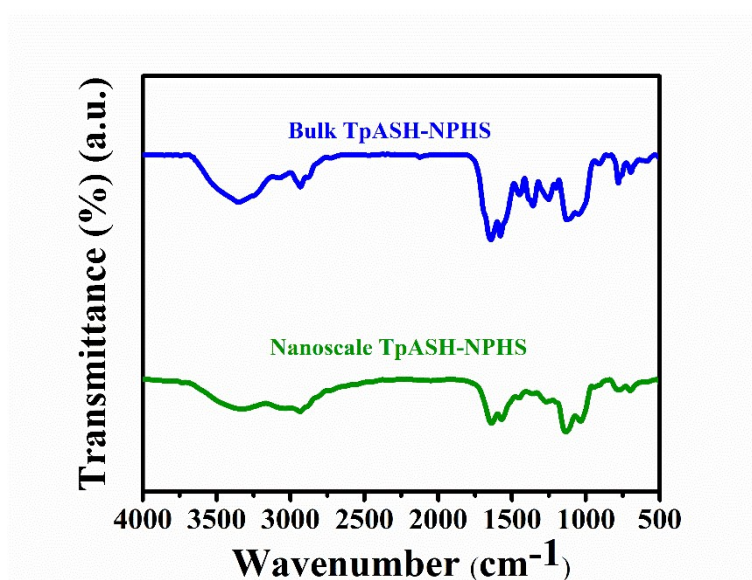
**Fig. S14.** Comparison of zeta potential between TpASH, TpASH-Glc and TpASH-NPHS.



**Fig. S15.** UV-vis spectra of TpASH, TpASH-Glc and TpASH-NPHS.

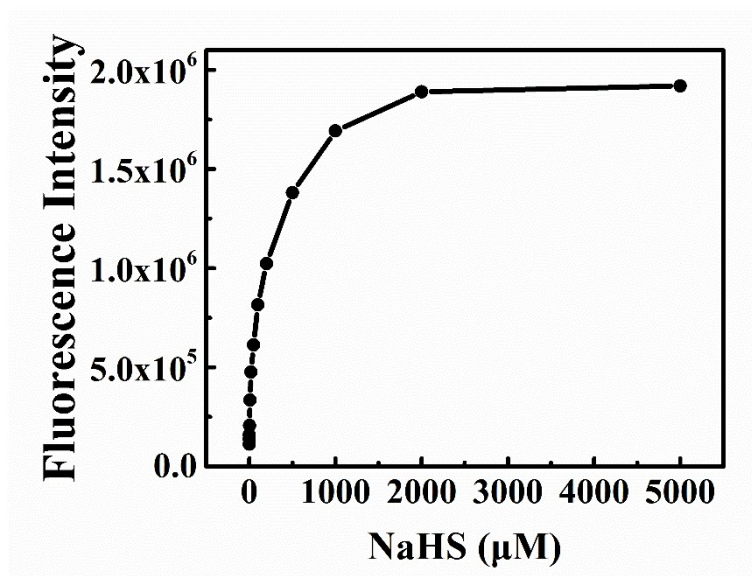


**Fig. S16.** AFM image of a) bulk TpASH-NPHS and b) nanoscale TpASH-NPHS. Comparison of average height profile between c) bulk TpASH-NPHS and d) nanoscale TpASH-NPHS as observed by AFM.

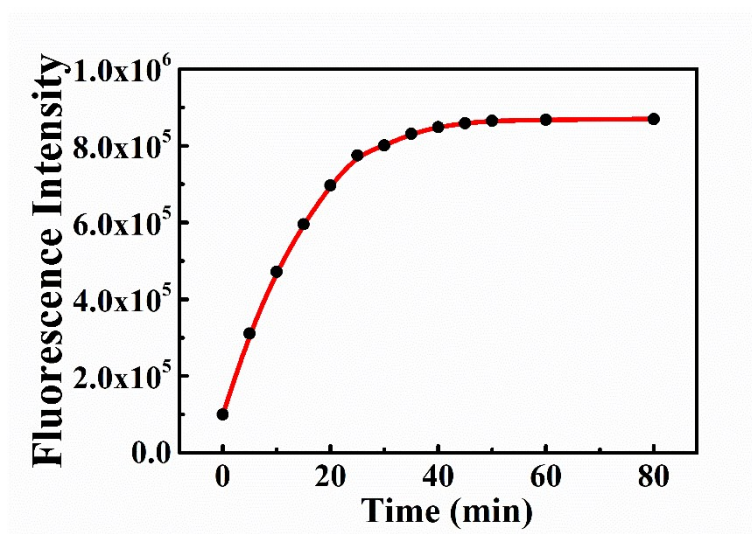


**Fig. S17.** Comparison of FTIR between bulk TpASH-NPHS and nanoscale TpASH-NPHS.

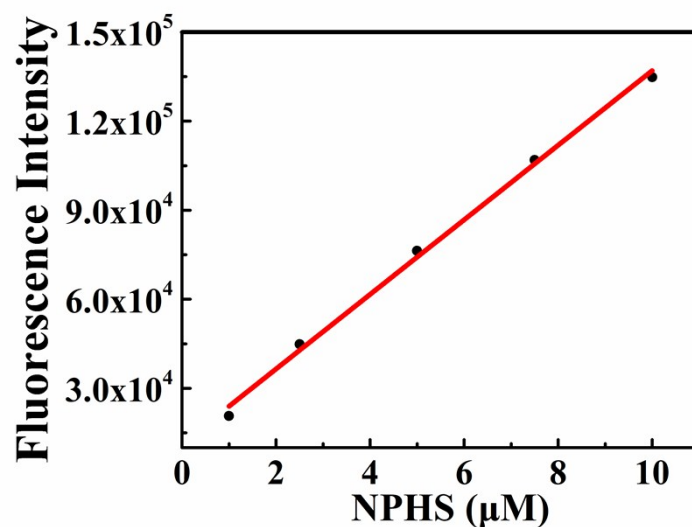
#### Section S-4: Biosensing of TpASH-NPHS



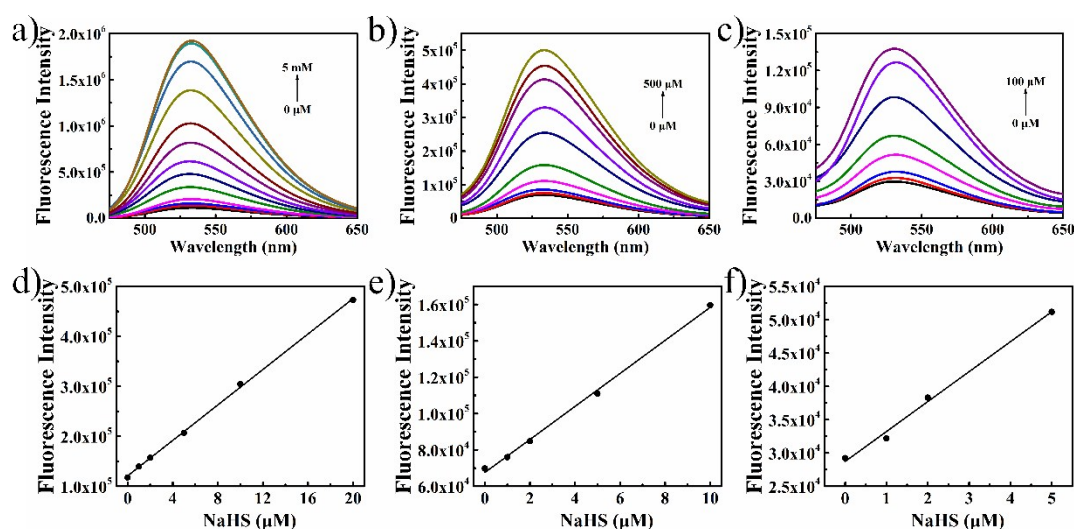
**Fig. S18.** The response curve of the TpASH-NPHS to NaHS. The curve was plotted with the fluorescence intensity vs. the NaHS concentration after incubation for 40 min.



**Fig. S19.** Time-dependent fluorescence response of TpASH-NPHS to NaHS (100  $\mu\text{M}$ ) in PBS (pH = 7.4).

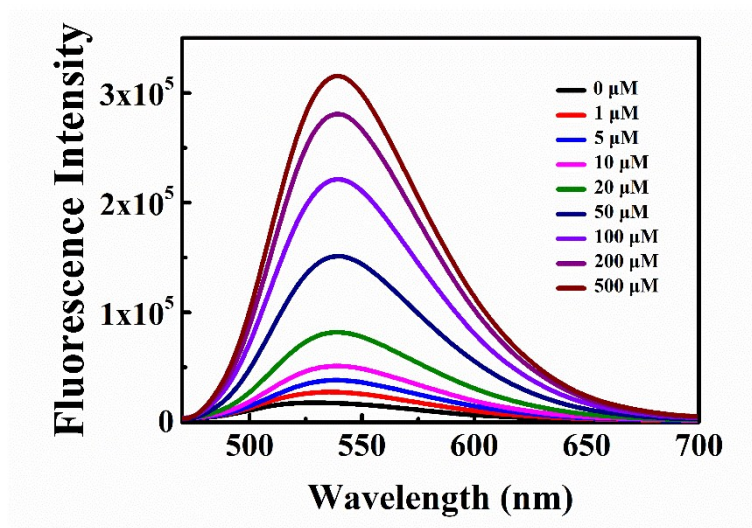


**Fig. S20.** The linear relationship between the concentration of NPBS and the fluorescence emission,  $y = 12562.78799x + 11385.90244$ ,  $R^2 = 0.996$ ,  $\lambda_{ex} = 440$  nm. The loading concentration of NPBS in TpASH-NPBS (100 μg/mL) was 1.05 μM.

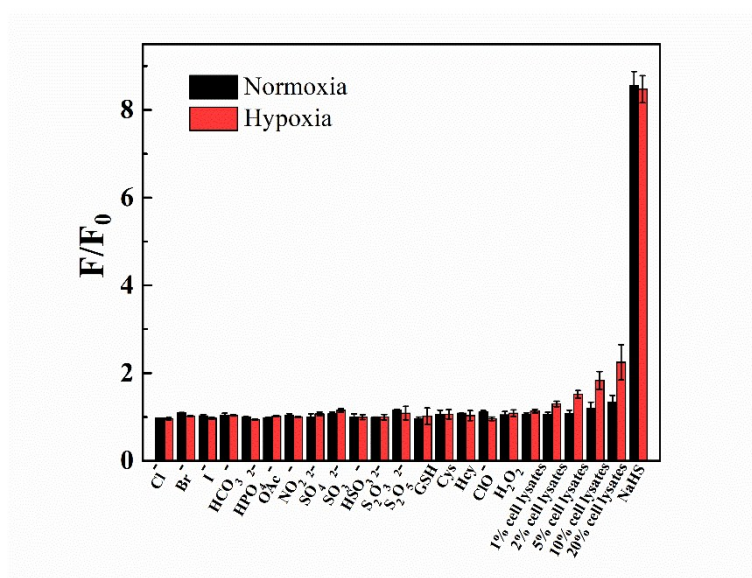


**Fig. S21.** (a-c) Fluorescence response of TpASH-NPBS with varied loading concentration of NPBS to NaHS. (d-f) Linear response of TpASH-NPBS to low NaHS concentrations. (a,d) The loading concentration of NPBS in TpASH-NPBS (100 μg/mL) was 1.05 μM. The detection limit was calculated to be 0.11 μM. (b,e) The loading concentration of NPBS in TpASH-NPBS (100 μg/mL) was 0.55 μM. The detection limit was calculated to be 0.45 μM. (c,f) The loading concentration of NPBS in TpASH-NPBS (100 μg/mL) was 0.20 μM. The detection limit was calculated to be 0.97 μM.

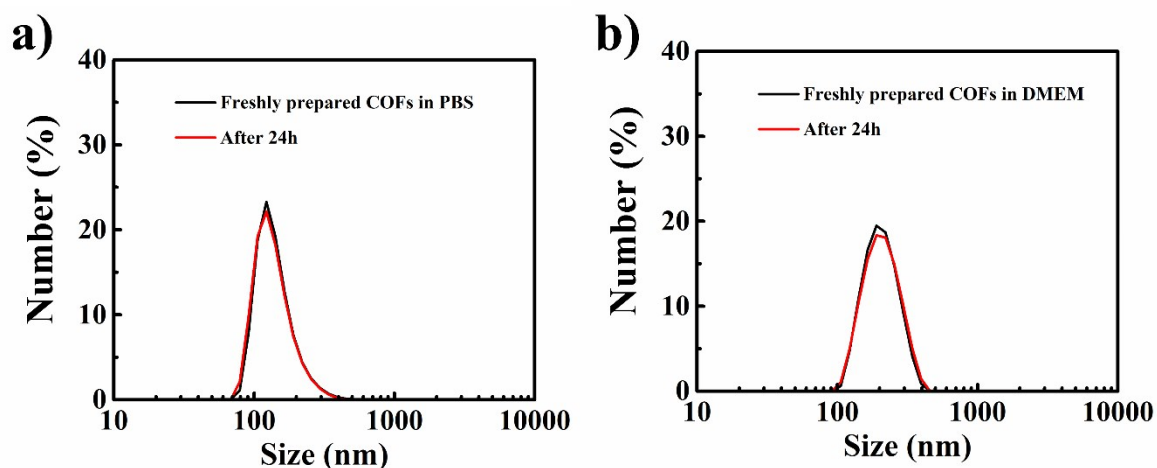




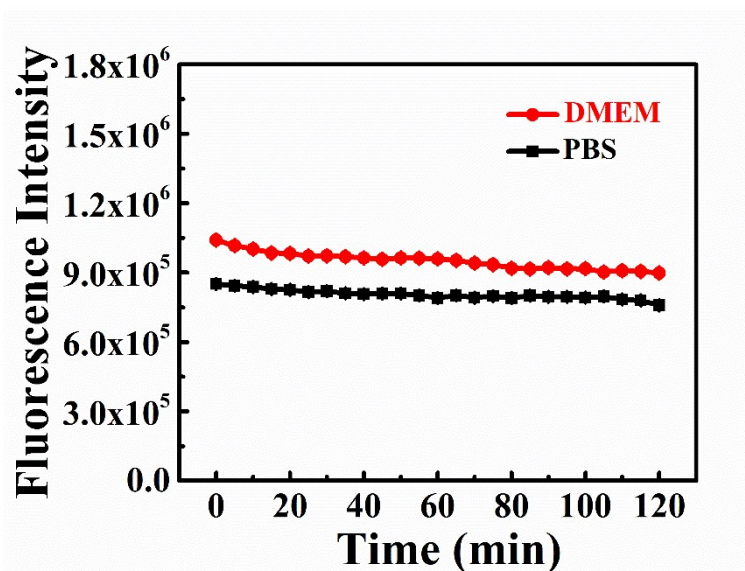
**Fig. S22.** The fluorescence emission spectra of CNPHS (10  $\mu\text{M}$ ) in the presence of different concentrations of NaHS in PBS (pH = 7.4) with 5% DMSO (v/v).



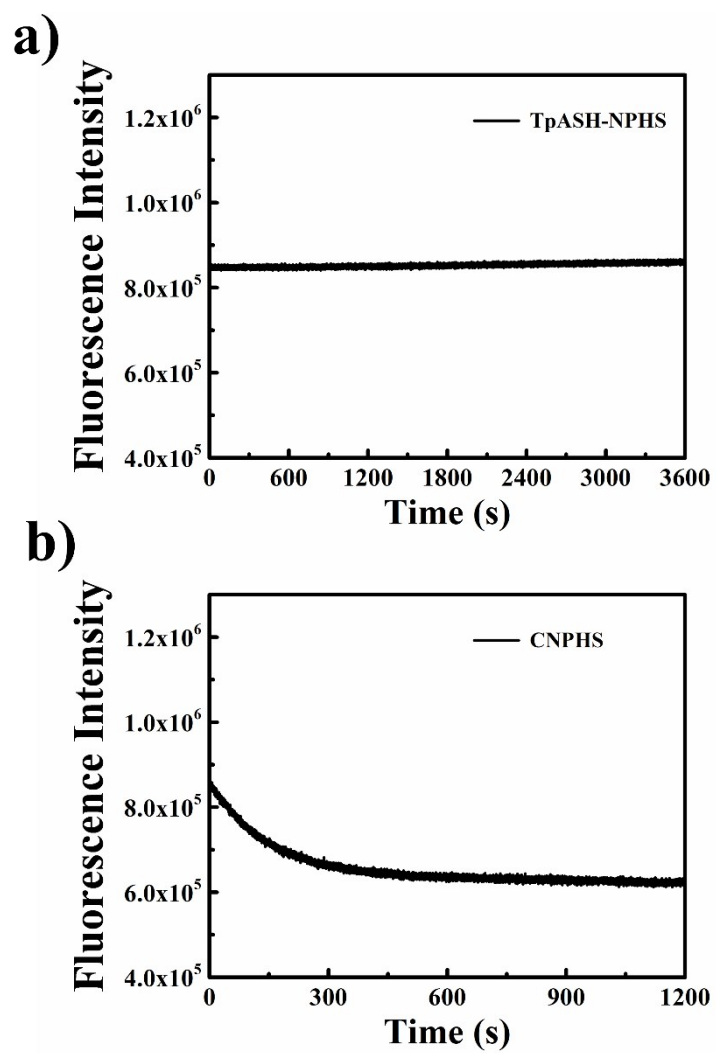
**Fig. S23.** Fluorescence response of CNPHS to various substances under normoxic (21%  $\text{O}_2$ ) and hypoxic (<0.1%  $\text{O}_2$ ) conditions.



**Fig. S24.** DLS size profiles of TpASH-NPHS in a) PBS (pH = 7.4) and b) DMEM-contained (50% v/v) solution.

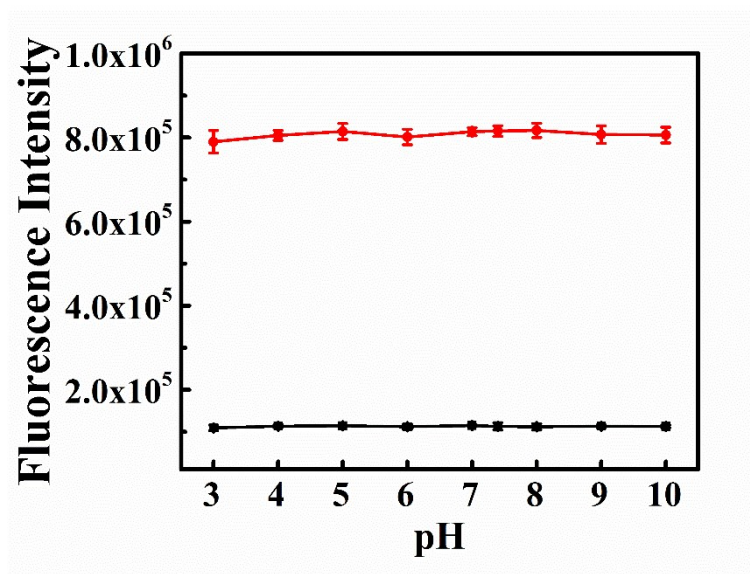


**Fig. S25.** Photostability of TpASH-NPHS with in PBS (pH = 7.4, black line) and DMEM-contained (50%, v/v) solution (red line) after additional NaHS by using 11 W LED lamp as the radiation source.

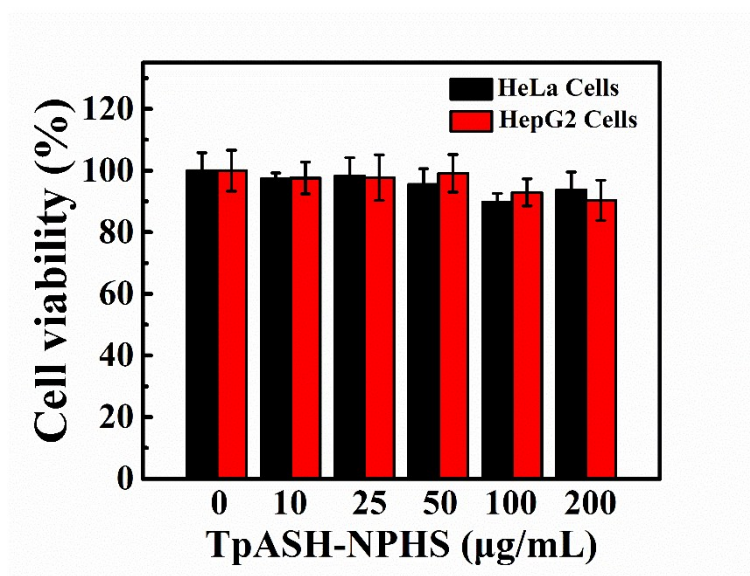


**Fig. S26.** Real-time fluorescence scanning of a) TpASH-NPHS (100  $\mu\text{g/mL}$ ) and b) CNPHS (5  $\mu\text{M}$ ) in PBS (pH = 7.4) with 5% DMSO (v/v) after additional NaHS.  $\lambda_{\text{ex}}$  = 405 nm,  $\lambda_{\text{em}}$  = 535 nm.

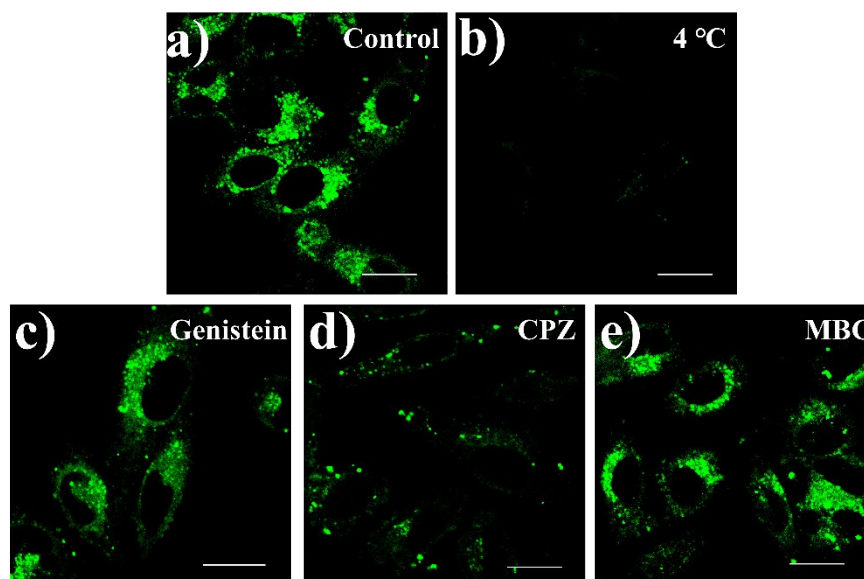




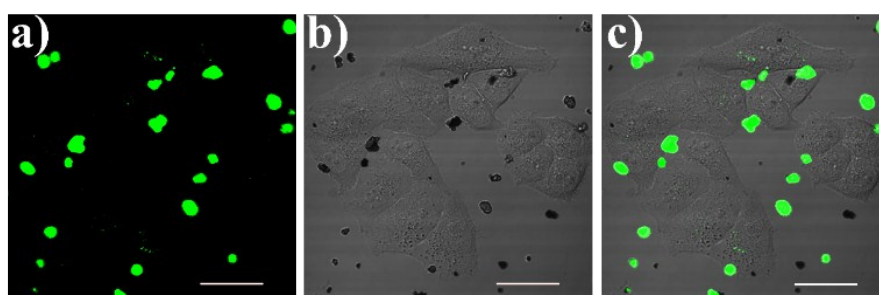
**Fig. S27.** The fluorescence intensity at 535 nm versus pH values for TpASH-NPHS (black line) and TpASH-NPHS after additional NaHS (red line).  $\lambda_{\text{ex}} = 440$  nm.



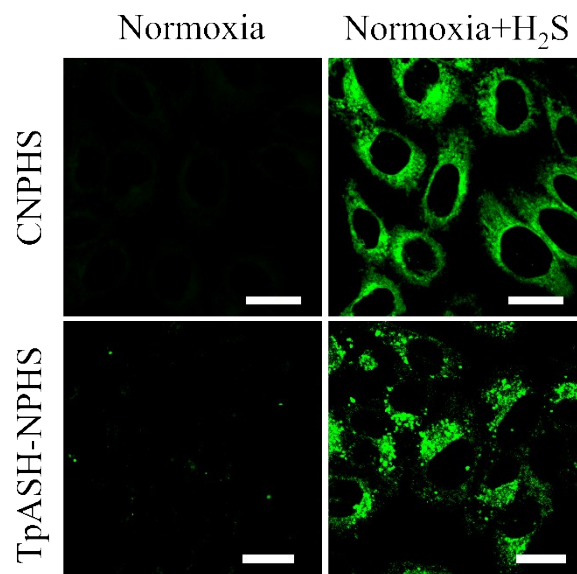
**Fig. S28.** Cytotoxicity assay of HeLa cells (black bar) and HepG2 cells (red bar) treated with different concentration of TpASH-NPHS.



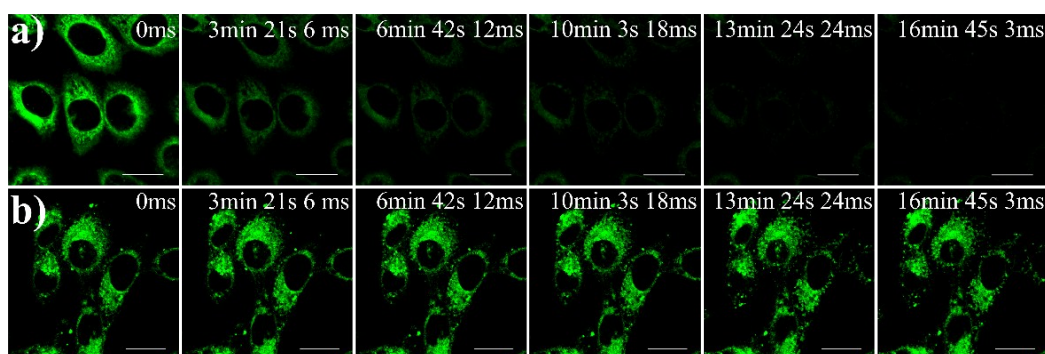
**Fig. S29.** Confocal fluorescence imaging of TpASH-NPHS in HepG2 cells under different conditions as indicated. a) control; b) 4 °C; c) genistein; d) CPZ; e) MBC. Scale bar: 20  $\mu$ m.



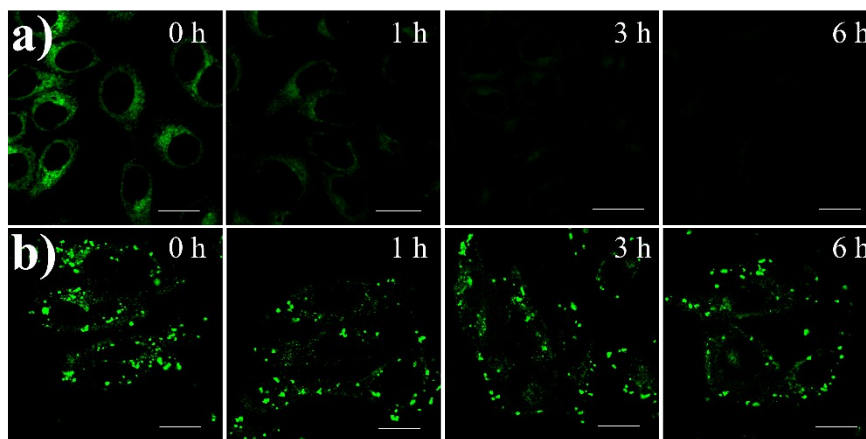
**Fig. S30.** Confocal fluorescence imaging of the bulk TpASH-NPHS (100  $\mu$ g/mL) in HeLa cells with NaHS (100  $\mu$ M). Scale bar: 20  $\mu$ m.



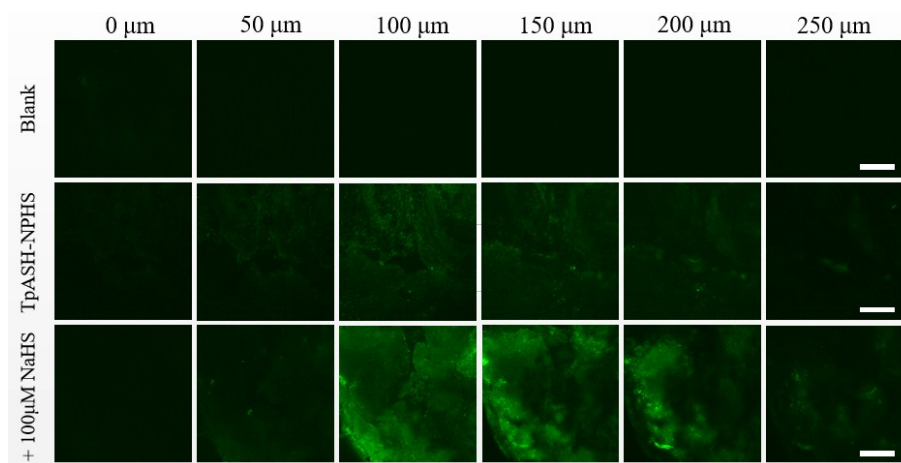
**Fig. S31.** Confocal fluorescence imaging of CNPHS or TpASH-NPHS in HepG2 cells under normoxic conditions with or without exogenous H<sub>2</sub>S added. Scale bar: 20  $\mu$ m.



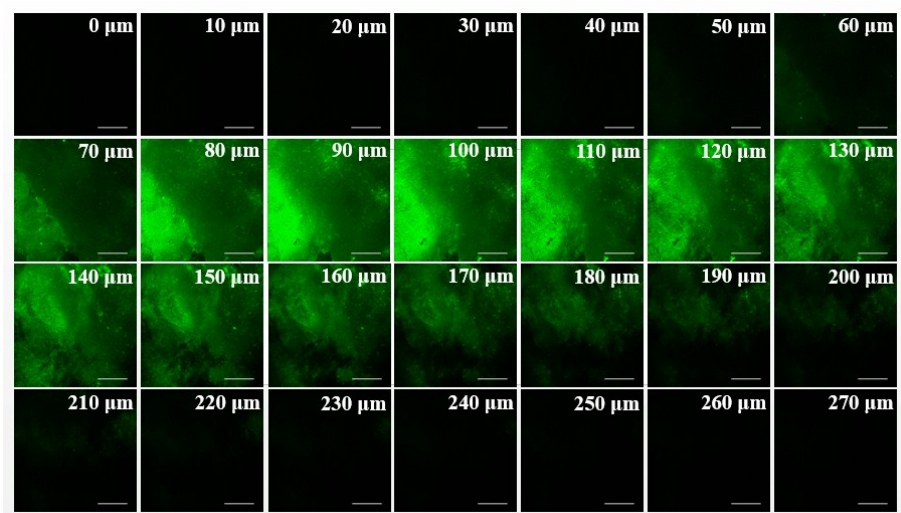
**Fig. S32.** Real-time confocal fluorescence imaging of a) CNPHS (1  $\mu$ M) and b) TpASH-NPHS (100  $\mu$ g/mL) after additional NaHS (100  $\mu$ M).  $\lambda_{\text{ex}} = 405$  nm,  $\lambda_{\text{em}} = 500$ -600 nm. Scale bar: 20  $\mu$ m.



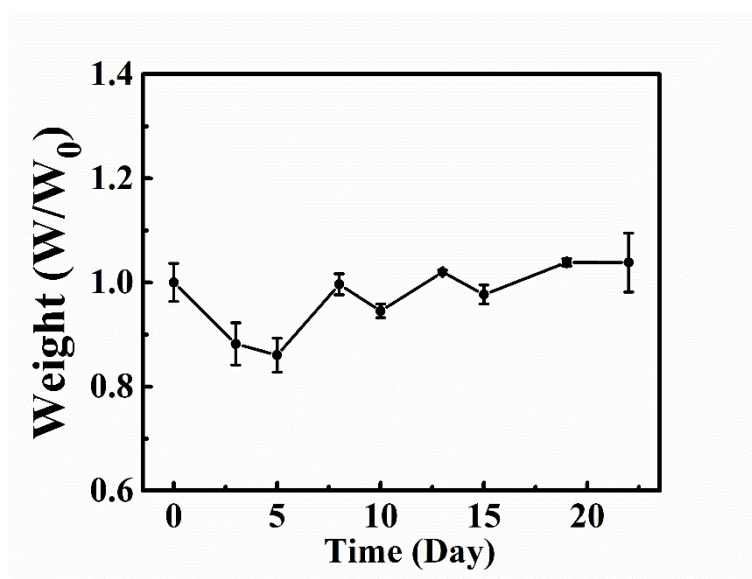
**Fig. S33.** Long-term cell tracing images of a) CNPHS and b) TpASH-NPHS after additional NaHS for designated time intervals including 1 h, 3 h and 6 h. Scale bar: 20  $\mu\text{m}$ .



**Fig. S34.** Depth fluorescence images of TpASH-NPHS (100  $\mu\text{g/mL}$ ) without or with NaHS (100  $\mu\text{M}$ ) in HeLa tumor tissues. The change of fluorescence intensity with scan depth were determined by spectral confocal multiphoton microscopy in the Z-scan mode (step size, 5  $\mu\text{m}$ ). Scale bar: 300  $\mu\text{m}$ .

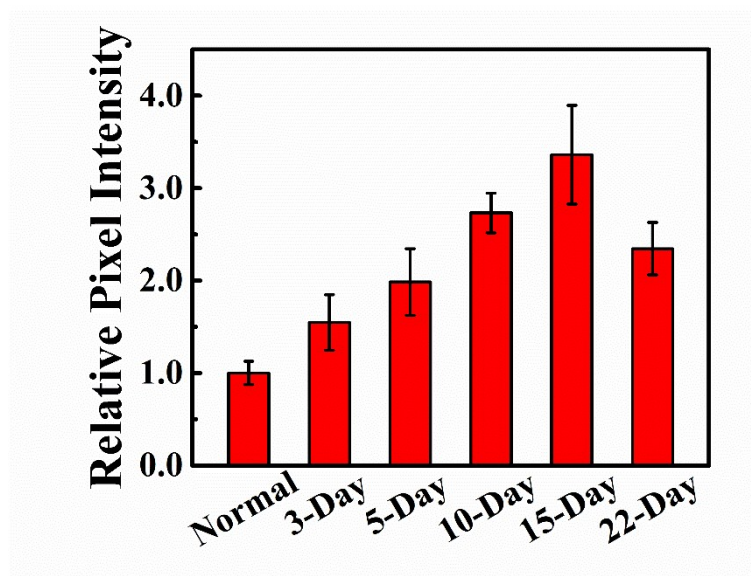


**Fig. S35.** Depth of TP fluorescence images of TpASH-NPHS (100  $\mu\text{g/mL}$ ) in HepG2 tumor tissues incubated with NaHS (100  $\mu\text{M}$ ) under hypoxia.  $\lambda_{\text{ex}} = 820 \text{ nm}$ . Scale bars: 300  $\mu\text{m}$ .

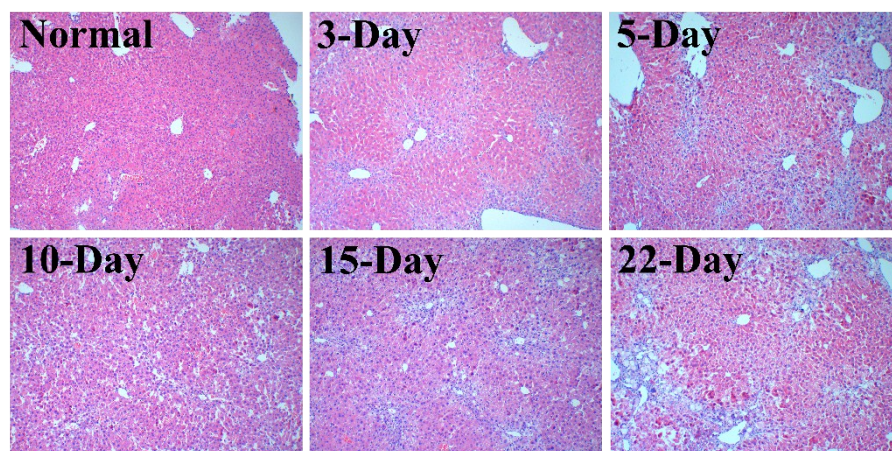


**Fig. S36.** The weights of cirrhotic mice at different  $\text{CCl}_4$ -treated periods. The weights of initial normal mice are defined as 1.0.





**Fig. S37.** The relative pixel fluorescence intensity of liver tissue images in cirrhotic mouse model at different CCl<sub>4</sub>-treated periods. The pixel signal intensity from TpASH-NPHS incubated with normal tissues is defined as 1.0.



**Fig. S38.** H&E staining images of liver tissues in cirrhotic mouse model at different CCl<sub>4</sub>-treated periods.

## References

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